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ANAEROBIC FERMENTATION OF CRYPTOSTEGIA LEAVES FOR RECOVERY OF RUBBER[1]

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Cryptostegia grandiflora R. Br. is a foliose tropical vine which bears a latex rich in rubber. The rubber can be obtained by cutting the long whiplike shoots or tapping the bark of the main trunk and collecting the latex which exudes. Recent work in this laboratory (Whittenberger, Brice, and Copley) has shown that the leaves of the plant also contain considerable quantities of rubber, which is localized in globules within the protoplasm of the chlorenchyma. This "cell rubber" comprises about 85 per cent of the total rubber of the leaves, the remaining 15 per cent being typical latex rubber, which is in the latex duct system of the leaf. The cell rubber cannot be recovered by tapping the latex system of the plant nor have satisfactory pebble-milling procedures been evolved analogous to those used for recovery of the rubber contained in the bark and wood of guayule. Solvent extraction is also unsatisfactory, as the yield of rubber is poor even after prolonged extraction unless the leaves are given an extensive chemical treatment prior to extraction.

Use of fermentation as an aid in the recovery of rubber from plants is not new. Lamb (1873) fermented milkweed prior to solvent extraction of the rubber, and Saunders (1875) found that a brief fermentation of *Asclepias cornuti* made the rubber more easily soluble. Aerobic fermentation of guayule prior to mechanical recovery of the rubber was patented by Spence (1933) and further investigated by Naghski, White, and Hoover (1944).

It therefore appeared probable that destruction of the cell walls by fermentation would make the rubber available for recovery. The conditions and procedure for this unique fermentation are discussed here. A report on aspects of the problem which bear on the recovery and technology of the rubber will be presented elsewhere (Hoover, Dietz, Naghski, and White).

EXPLORATORY FERMENTATIONS

The fresh living leaves used in these preliminary experiments were from an F_1 hybrid of *Cryptostegia madagascariensis* × *C. grandiflora*.[3] The material

[1] Natural rubber from domestic sources. Paper no. 7.

[2] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

[3] This material was obtained from the U. S. Plant Introduction Garden, Coconut Grove, Florida, through the courtesy of the Bureau of Plant Industry, Soils, and Agricultural Engineering, United States Department of Agriculture.

used in the remainder of the fermentation studies was dried leaves of *C. grandiflora*.[4]

Among the microorganisms tested were *Chaetomium globosum*, *Trichoderma lignorum*, *Aspergillus fumigatus*, *Penicillium* sp., *Aspergillus* spp., *Clostridium* sp.,[5] *Clostridium roseum*,[5] *Clostridium felsineum*,[5] *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Flavobacterium fecale*, and thermophilic cellulose fermenters. Several of these attacked the cell walls slowly, but two—*C. roseum* and *C. felsineum*—fermented the leaves readily, with considerable production of gas. Mild agitation disintegrated the fermented leaves and liberated a finely divided material which settled out as a green sediment. This sediment consisted of the coagulated contents of chlorenchyma cells devoid of cell walls, with the rubber globules embedded within them. Boiling with dilute alkali dissolved the protoplasts and liberated the rubber globules, which then rose to the surface. The rubber was also recovered readily from the protoplasts by solvent-extraction procedures. Photomicrographs of cross sections of a fresh living leaf, the isolated contents of the chlorenchyma cell, and the rubber-bearing globules recovered therefrom are shown in figure 1.

Clostridium roseum was selected for the investigation.

EFFECT OF VARIABLES ON THE FERMENTATION

Assay method. The effects of time, temperature, concentration of leaves, pretreatment of leaves, added nutrients, and condition of culture on the rate of retting were studied to determine optimum conditions for retting. The following method of assay was developed to evaluate the effects of these variables. Twenty-five grams (dry weight) of leaves in a 1.5-liter Erlenmeyer flask were given the desired pretreatment and made up to the desired volume with a mineral salt solution (Allison and Hoover, 1934) or water, and then inoculated with a 10 per cent volume of an 18-hour broth culture. The flask was equipped with a water trap to permit flushing out with nitrogen and also to allow the escape of fermentation gases. During incubation, it was shaken occasionally to keep "heading" at a minimum. Except for the temperature studies, all assays were incubated at 39 to 40 C. At the end of incubation, the contents of the flask were transferred to a 1-quart fruit jar and shaken for 30 minutes in a mechanical shaker. The disintegrated leaves were screened on a 20-mesh sieve. The residue was diluted with 200 ml of water and screened again. The residue on the screen was dried at 100 C and weighed. The relative efficiency of the fermentation, expressed as "degree of retting," was calculated as follows:

$$\text{Degree of retting} = \frac{\text{g of residue from standard treatment}}{\text{g of residue from experimental treatment}}$$

The weight of residue from leaves extracted for three 5-minute periods with boiling water, made up to 5 per cent concentration, inoculated, and incubated

[4] This material was obtained through the courtesy of the Atkins Institution of the Arnold Arboretum of Harvard University, Soledad, Cuba.

[5] Cultures of *C. roseum* A 42, *C. felsineum* A 41, and *C.* sp. A 40 were obtained through the courtesy of Dr. E. McCoy of the University of Wisconsin.

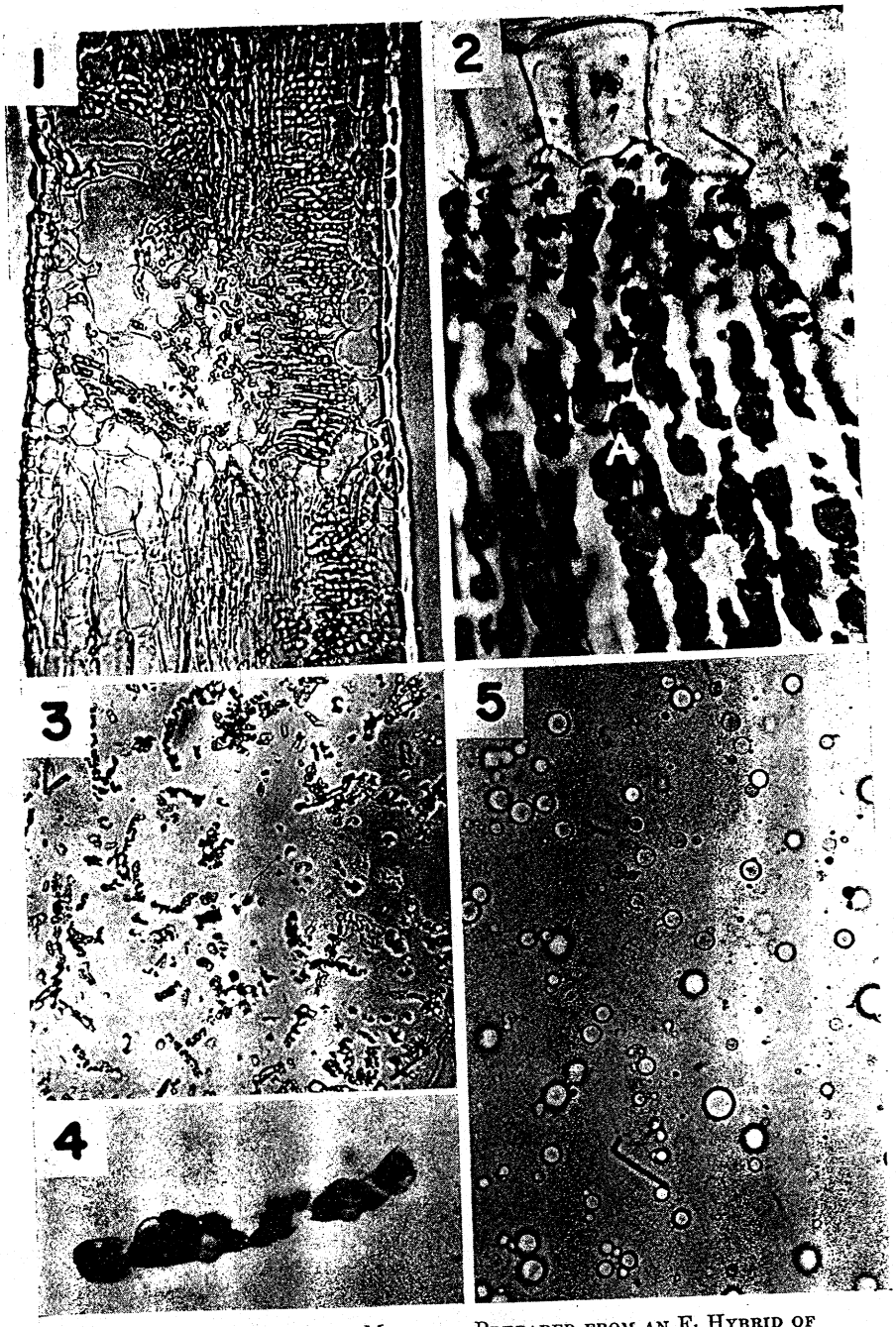

FIG. 1. PHOTOGRAPHS OF MATERIAL PREPARED FROM AN F_1 HYBRID OF C. MADAGASCARIENSIS × C. GRANDIFLORA

1. Cross section of preserved specimen of mature leaf; unstained. 130 ×. Rubber globules visible in the chlorenchyma.
2. Cross section of fresh, living, senescent leaf. 460 ×. (A) Rubber-bearing globules and chloroplasts within the palisade cells. (B) Upper epidermis.
3. Protoplasts from retted leaves. 130 ×.
4. A protoplast from retted leaf. 1000 ×.
5. Suspension of rubber-bearing globules isolated from protoplasts by alkaline digestion of the protoplasts. 460 ×. Many of the globules are not in focus.

at 39 to 40 C for 2 days was taken as the standard. This weight depended upon the particular batch of leaves fermented; consequently controls were run as part of each experiment. Leaching out soluble constituents during pretreatment and incubation of the uninoculated control produced a loss in weight equivalent

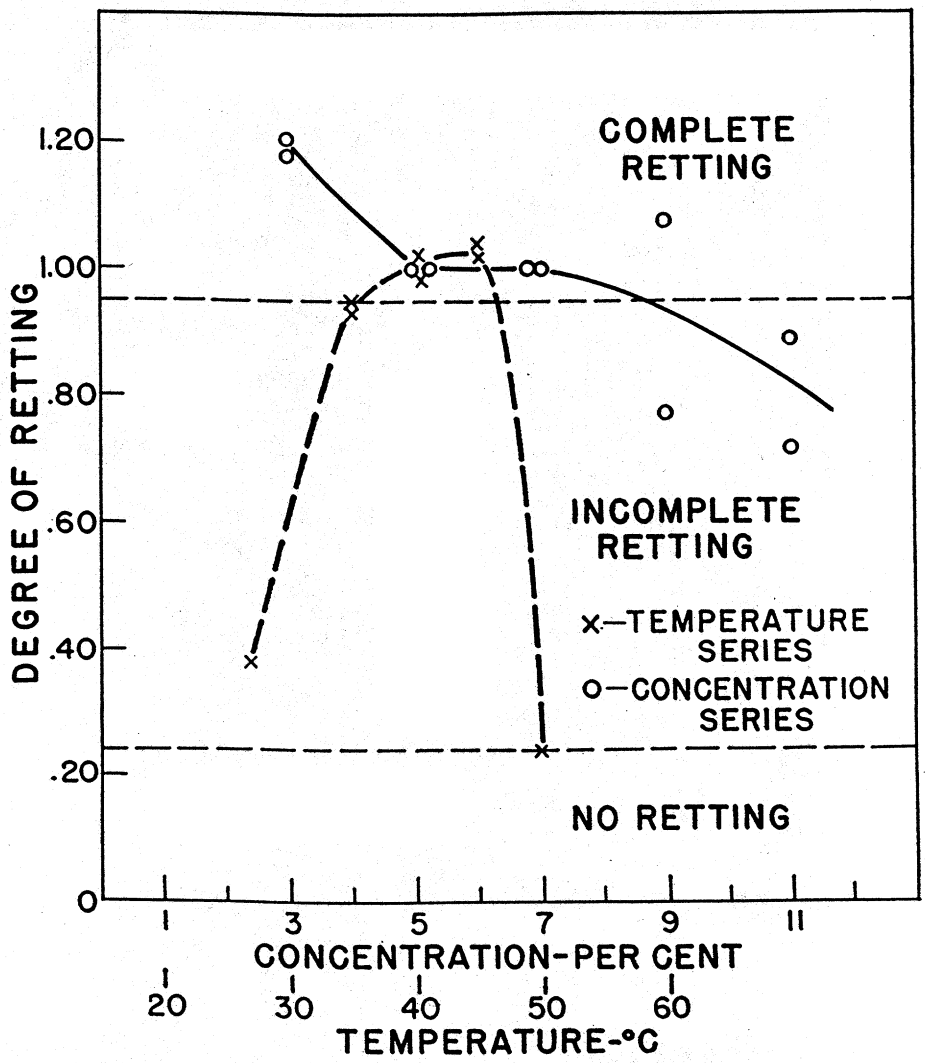

FIG. 2. EFFECT OF TEMPERATURE AND CONCENTRATION ON EXTENT OF RETTING OF CRYPTOSTEGIA LEAVES BY CLOSTRIDIUM ROSEUM IN TWO DAYS AT 39 TO 40 C

to a degree of retting of 0.24; therefore values below this point represent no retting. Microscopic examination of samples giving a value of 0.95 or above showed that retting was essentially complete. This assay method enabled us to evaluate the effect of a large number of factors upon the fermentation and quickly determine optimum conditions. The excellent reproducibility of results is shown later in figure 2.

Production of inoculum. A satisfactory medium for culturing the inoculum consisted of 16 ml of blackstrap molasses, 1 g of glucose, 5 ml of corn steep liquor, and 1 g $(NH_4)_2SO_4$ per liter of mineral salts solution[6] (Allison and Hoover, 1934); the reaction was adjusted to pH 6.8 to 7.2 with 10 per cent sodium hydroxide. No attempt was made to simplify this medium further. Growth was rapid; 12- to 18-hour cultures gave satisfactory inocula for fermentations. Inocula more than 24 hours old became sluggish and showed a prolonged lag period. Microscopic examination of the latter showed spores to be predominant. The cultural and biochemical characteristics of *C. roseum* have been reported by McCoy and McClung (1935).

Subculturing. During exploratory work on the decomposition of *Cryptostegia* leaves, the culture was maintained in an actively growing state by transferring it to a fresh tube of corn mash every other day rather than starting a fresh culture from the dry-soil spore suspension. After a month of such treatment the culture

TABLE 1

Effect of subculturing C. roseum on rate of retting of Cryptostegia leaves

AGE OF CULTURE FROM STOCK*	NUMBER OF CONSECUTIVE SUBCULTURES IN CORN MASH	DEGREE OF RETTING AFTER	
		2 days	3 days
days			
2	1	1.00	1.10
44	3	0.90	1.04
44	5	0.62	0.76
13	7	0.59	0.76
44	22	0.58	0.58

* Stock culture was a spore suspension in dry sterile soil.

fermented the leaves poorly. Table 1 gives data from an experiment designed to show the effect of frequency of transferring *C. roseum* in corn mash on the loss of retting ability. A culture freshly initiated from the dry-soil spore suspension produced complete retting in 2 days. Cultures passing through more than the initial transfer gave progressively lower retting values. Cultures permitted to go into the spore state did not continue to lose retting ability, as shown by the 44-day cultures that sporulated in the corn mash after the third and fifth transfer.

However, a stock culture maintained in the spore state in dry sterile soil retained its activity and viability for more than 18 months. McCoy *et al.* (1926) reported that cultures developed from soil preparations were more active than those from corn mash.

Pretreatment of the leaves. The effects of various leaching procedures and of the medium are presented in table 2. Excellent results were obtained when the leaves were given three 5-minute extractions or one 15-minute extraction with

[6] The solution contained 0.3 g KH_2PO_4, 0.7 g K_2HPO_4, 0.2 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.1 g $CaSO_4 \cdot 2\,H_2O$ per liter.

boiling water. Removal of extractives increased the rate of retting, presumably owing to the removal of inhibiting materials. It was also found that the leached leaves were fermented equally well whether mineral salts solution or tap water was used as the medium. Apparently sufficient minerals and nutrilities for optimum growth were carried over in the inoculum. Therefore, tap water was used in all large-scale experiments.

Concentration of solids. The maximum concentration of leaves which could be fermented in 2 days was determined. The results are shown in figure 2. The reproducibility of data obtained at 3, 5, and 7 per cent concentrations (based on original unleached weight of dry leaves) is illustrated by the superposition of

TABLE 2

Effect of pretreatment and medium on degree of retting of Cryptostegia leaves by C. roseum

CONCENTRATION OF LEAVES	PRETREATMENT	MEDIUM	DEGREE OF RETTING
%			
3	1 60-min steep at 62 C, not drained	tap water	0.66
3	1 60-min steep at 62 C, drained	mineral salts	0.74
3	1 60-min steep at 62 C, drained	tap water	0.76
5	3 20-min steeps at 55 C, drained	mineral salts	0.74
5	1 1-min boil, drained	mineral salts	0.84
5	1 5-min boil, drained	mineral salts	0.90
5	1 10-min boil, drained	mineral salts	0.95
5	1 15-min boil, drained	mineral salts	1.00
5	1 15-min boil, not drained	tap water	0.77
5	2 5-min boils, drained	mineral salts	0.96
5	3 5-min boils, drained (standard treatment)	mineral salts	(1.00)
3	3 5-min boils, drained	mineral salts	1.00
7	3 5-min boils, drained	mineral salts	1.00
3	4 5-min boils, drained	mineral salts	1.05
3	4 5-min boils, drained	tap water	1.00

points for different experiments. Complete fermentation of the 7 per cent slurry was obtained. The inconsistent results at 9 and 11 per cent concentrations were due to the difficulty encountered in keeping the leaves submerged. Unless the leaves were submerged they fermented slowly. In large-scale experiments concentrations above 5 per cent were difficult to handle with the equipment available. Undoubtedly the fermentation could be carried out with a higher solids content in equipment designed for the purpose.

Temperature. Temperature had a marked effect on the fermentation (figure 2). Retting was substantially complete in 2 days at 35 to 45 C. At 28 C the decomposition proceeded slowly; and at 50 C no retting occurred. McCoy and McClung (1935) found that the range of temperature at which this organism

grew was from 8 to 62 C. They also found that the fermentation of corn mash at the higher temperatures investigated (54 to 62 C) was incomplete, and, of course, at low temperatures growth was slow.

Contaminants. *C. roseum* established an essentially pure culture when incubated anaerobically, even with unsterilized leaves. It is therefore not especially sensitive to bacterial competition. This is in agreement with the results obtained by Dr. E. McCoy in retting plants for bast fibers with this organism (personal communication).

Action antagonistic to *C. roseum* was observed when attempts were made to ret a sample of molded leaves. Growth of bacteria was light, and the cell walls of the leaves were not decomposed.

FERMENTATION PROCEDURE

On the basis of the foregoing results, large-scale fermentations were carried out in 40- and 120-gallon volumes. Many runs were made to prepare sufficient material for chemical and physical tests. A typical run will be described in which the leaves were retted in a 50-gallon barrel under nearly optimum conditions. A 7.45-kg lot of dry leaves was extracted with boiling water, made up to 140 liters with tap water (40 C), and inoculated by introducing 10 liters of an 18-hour culture of *C. roseum* at the bottom. The barrel was then covered with a gasketed lid and kept anaerobic by leading in carbon dioxide gas generated from solid carbon dioxide in a Dewar flask. Incubation was carried out at 35 to 37 C for 2 days. Difficulty with "heading" was overcome by using a motor-driven, low-pitch propeller blade, which just swept the surface of the liquor and revolved at 6 rpm. This intermittently submerged the leaves that were pushed out of the liquid by the gases and also produced a gentle rocking action, which dislodged the gas and permitted it to escape.

After two days' incubation, the leaves were well digested and disintegrated. The slurry was passed over a vibrating screen (80 by 80 meshes to the inch). The liquid and the protoplasts passed through, but the bagasse (cuticle, veins, and small stems) remained on top. Because the latex ducts in *Cryptostegia* leaves are long and closely associated with the veins, they were trapped in the bagasse. The bagasse was dispersed in water to half the original volume and again screened to recover the protoplasts that were trapped mechanically. The bagasse was freed of excess water by pressing and then dried. The protoplasts (sp gr 1.17 to 1.27) were recovered from the liquor in which they were suspended by gravity settling and decantation; a slurry containing about 4 to 7 per cent solids was obtained. The slurry was further freed of soluble materials by diluting with water, settling, and decanting.

The data presented in table 3 show that the protoplasts amounted to one fourth of the original weight of leaves, and contained more than three fourths of the rubber, whereas the bagasse fraction amounted to less than one eighth of the original weight and contained less than one fourth of the rubber. Microscopic examination showed that the protoplasts were essentially free of latex ducts. Since the bagasse fraction included a small portion of incompletely dis-

"The disintegration of tissue here differs from reaction of *C. acetobutyllicum* and suggests fermentation of pectin or cellulose or both." Therefore, to correlate microscopic observations with chemical analysis, samples of leaves were analyzed before and after fermentation for 4 days. The residue after fermentation was washed thoroughly to remove soluble substances, and the total insoluble fraction was recovered quantitatively. For comparison another *Clostridium* culture (A 40) which does not produce satisfactory retting was used in a similar experiment. Determinations of crude hemicellulose and crude cellulose were

TABLE 4

Effect of concentration of alkali and protoplasts on rate of solution of protoplasts

PROTOPLAST CONCENTRATION	CONCENTRATION OF SODIUM HYDROXIDE IN LIQUOR	TIME OF BOILING*	RATIO OF PROTOPLASTS TO SODIUM HYDROXIDE
%	%	*min.*	
1.6	0.3	10	5.3
5.0	0.5	15	10
5.0	1.0	5	5
10.0	1.0	20	10
10.0	1.5	15	6.6

* Minimum time necessary to effect complete dissolution of protoplasts as observed microscopically.

TABLE 5

Composition of leaves before and after retting for four days with C. roseum and with Clostridium A 40

CONSTITUENT	BEFORE RETTING	AFTER RETTING BY C. ROSEUM		AFTER RETTING BY CLOSTRIDIUM A 40	
		%	% LOSS	%	% LOSS
	%				
Dry weight	100	38.5	61.5	53.0	47.0
Ash	16.96	4.84	89.1	5.59	82.6
Nitrogen	2.67	5.35	22.9	4.46	11.2
Crude hemicellulose	2.65	2.70	61.1	2.53	49.5
Crude cellulose	9.25	6.03	75.0	9.96	43.1

made before and after fermentation. Results of the analyses are presented in table 5. Ash and nitrogen were determined by the methods of analysis of the Association of Official Agricultural Chemists for plant materials (1940). The crude hemicellulose was determined by a modification of the methods of Potts and Bridge (1937); the crude cellulose was determined by a modification of the method used by Vladesco (1940). Before these methods could be applied, the plant had to be freed of rubber and pectin by digestion with ammonium oxalate followed by successive extractions with toluene, alcohol, and benzene.

Although the fermentation with both organisms proceeded rapidly, with vigorous evolution of gas, the analytical results, together with the differences in

integrated leaves and also mechanically held some released protoplasts, it contained an appreciable quantity of cell rubber. These results are in agreement with those of Whittenberger, Brice, and Copley, who found about 10 to 15 per cent of the total rubber of the leaves to be in the latex-duct rubber fraction. The loss in dry weight effected by the fermentation increased the over-all concentration of rubber from 4.2 to 11.2 per cent. The rubber was determined by a method developed at this laboratory (unpublished).

TABLE 3

Distribution of rubber in protoplasts and bagasse of Cryptostegia leaves fermented by C. roseum

	ORIGINAL LEAVES	PROTOPLASTS	BAGASSE	RETTING LOSS
Dry weight, kg	7.45	1.86	0.87	4.72
% of original leaves		25.0	11.7	63.3
Rubber content, %*	4.2	12.7	8.1	
% of original rubber in—		76.0	22.6	1.4

* Moisture-free basis

DIGESTION OF PROTOPLASTS

In addition to the rubber and resin the dried protoplasts contained 5.5 to 7.3 per cent nitrogen (depending on the composition of original leaves), equivalent to 34 to 45 per cent protein. Considering that the protoplasts are proteinaceous, it would appear that they should be decomposed by microorganisms or proteolytic enzymes. They proved, however, to be refractory. Attempts to digest the protoplasts with papain were not successful. When a dilute suspension of protoplasts was first adjusted with NaOH so that after boiling or autoclaving at 15 pounds for 20 minutes the pH was between 8.0 and 8.5, the rubber globules were liberated by fermentation with *P. aeruginosa*, *F. fecale*, and *B. subtilis*. Adjusting the reaction with alkali did not liberate the rubber globules but changed the structure of the protoplasts so that these bacteria were able to digest them. Hydrochloric, sulfuric, and oxalic acids in concentrations up to 10 per cent did not digest the protoplasts (10 per cent suspension), even after 5 hours' boiling, and a 40 per cent solution of urea also did not dissolve them to any appreciable extent. Boiling with dilute aqueous alkali (NaOH or KOH) dissolved the protoplasts, however, and released the rubber globules. The concentrations of dilute aqueous alkali and of protoplasts which permitted dissolution of the protoplasts are shown in table 4.

FERMENTATION OF CELLULOSE BY CLOSTRIDIUM ROSEUM

Microscopic examination of retted leaves showed that the parenchyma cell walls were digested and the protoplasts liberated, indicating that *C. roseum* was capable of fermenting cellulose. McCoy and McClung (1935) found that *C. roseum* did not ferment filter paper cellulose in tryptone broth. We have confirmed this result. However, concerning growth on potato slants, they state:

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Although the fermentation with both organisms proceeded rapidly, with vigorous evolution of gas, the analytical results, together with the differences in

the effectiveness of release of the protoplasts, allow certain distinctions to be made. *C. roseum* produced a definitely greater loss in weight, nitrogen, and crude cellulose than did *Clostridium* A 40. Both organisms caused about the same loss in crude hemicellulose. The protoplasts were not released by fermentation with culture A 40, even when the leaves were agitated mechanically. It appears therefore that the digestion of hemicellulose and the more readily available cellulosic materials does not permit liberation of the protoplasts from the cells. Apparently *C. roseum* attacks the structural cellulose of the parenchyma cell wall, thus releasing the coagulated cell contents. However, it does not decompose the cell walls of the veins and midribs, thus leaving a "skeleton" of the vascular system.

DISCUSSION

The voluminous literature dealing with biochemical decomposition of cellulose, hemicelluloses, etc., has been reviewed by Thaysen and Bunker (1927), by Norman (1937), and by Waksman (1932, 1940). Buswell and Hatfield (1939) have discussed the use of anaerobic fermentation for the production of combustible gas from cellulose and related constituents of agricultural residues. To our knowledge, fermentation has not been used for the segregation of the chlorenchyma cell contents.

The digestion of the cell wall, together with the chemical data obtained, strongly suggests that the cellulosic materials and related constituents of the cell wall were attacked. Moreover, it is well known that the retting of plants for bast fibers by related organisms has to be closely controlled to prevent overretting and consequent loss of strength of the fiber. It is apparent that after the pectinaceous substances have been decomposed, the organism gains access to the cellulosic fibers, thereby weakening them by partial digestion. The fact that the retting organisms have not been considered as having cellulose-digesting ability further indicates that action on filter paper may be too exclusive a criterion. Fuller and Norman (1943) have shown that cornstalk cellulose, especially when it contains xylans, is more readily utilized than filter paper and is vigorously decomposed by organisms that utilize filter paper slowly. McClung (1943) has also shown that the chromogenic anaerobes, especially of the *C. felsineum* type, are prevalent in nature and can be readily demonstrated by proper techniques. Our data further emphasize the possibility that this group of organisms plays a more important role in the decomposition of plant materials in nature than has been hitherto suspected.

Application of this unique fermentation in the study of other problems is at once apparent. It has already been utilized by Whittenberger, Brice, and Copley to prepare leaf skeletons in their study of the distribution of rubber in *Cryptostegia* leaves; and similar skeletons of other leaves have been prepared. In the separation and chemical study of the protoplasts from leaves of various plants now under investigation, the techniques used here have been applied without modification.

SUMMARY

Anaerobic decomposition of *Cryptostegia* leaves for the recovery of rubber was studied. A two-day fermentation of preboiled *Cryptostegia* leaves by *Clostridium roseum* at 35 to 45 C effected a loss of 60 per cent in the original dry weight of leaves and a 75 per cent loss in the crude cellulose. Consequently, the rubber content of the product was more than two and one-half times that of the original material, on a moisture-free basis. Parenchyma cell protoplasts were liberated by destruction of their cell walls. Screening fermented leaves sufficed to separate the protoplasts containing the cell rubber from the latex rubber in the other fraction comprising veins, epidermis, and cuticle.

The protoplasts were resistant to action by acids, but were dissolved by dilute alkali; the liberated rubber globules rose to form a cream.

It has been established microscopically and chemically that *C. roseum* ferments the cellulosic fractions of leaves *in situ*.

ACKNOWLEDGMENT

The authors wish to thank Dr. C. O. Willits and associates of this laboratory for developing chemical analytical methods and conducting the chemical analyses reported in this paper.

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